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The Patent Office

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1. Your reference — JWJ01060GB

2. Patent application number *(The Patent Office will fill in this part)* — 0317343.2

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

MEDICAL BIOSYSTEMS LTD.
The Old Mill
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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UK

07258635001

4. Title of the invention — POLYNUCLEOTIDE SEQUENCING

5. Name of your agent *(if you have one)*

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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Claim *(s)*	1 CF
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Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature: Gill Jennings & Every

Date: 24 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

JAPPY, John William Graham
020 7377 1377

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POLYNUCLEOTIDE SEQUENCING

Field of the Invention

The present invention relates to methods for determining the sequence of a polynucleotide as well as the detection of variations between sequences.

Background of the Invention.

The ability to determine the sequence of polynucleotide is a great scientific importance. Recently the Human Genome Project determined the entire sequence of the human genome (3*10 9) bases. This sequence information represents that of an average human. However, there is still considerable need and interest to understand the difference between individuals in a population at genetic level.

The principle method in general use for large-scale DNA sequencing is the chain termination method. This method was first developed by Sanger and Coulson (Sanger et al. Proc. Natl. Acad. Sci. USA 1977; 74: 5463-5467), and relies on the use of dideoxy derivatives of the four nucleoside triphosphates which are incorporated into the nascent polynucleotide chain in a polymerase chain reaction. Upon incorporation, the dideoxy derivatives terminate the polymerase reaction and the products are then separated by gel electrophoresis and analysed to reveal the position at which the particular dideoxy derivative was incorporated into the chain.

Although this method is widely used and produces reliable results, it is recognised that it is slow, labour-intensive and expensive.

Nucleic acid arrays have been used to determine polynucleotide sequences and SNPs, usually in the context of hybridisation events (Mirzabekov, Trends in Biotechnology (1994) 12:27-32). Many of these hybridisation events are detected using fluorescent labels attached to nucleotides, the labels being detected using a sensitive fluorescent detector, e.g. a charge-coupled detector (CCD). The major disadvantage of these methods is that repeat sequences can lead to ambiguity of results. This problem is recognised in Automation Technologies for Genome Characterisation, Wiley-Interscience (1997), ed. T.J.Beugelsdijk, Chapter 10:205-225.

Summary of the Invention

The present invention is based on the realisation that the kinetics of the template-primer duplex/complementary nucleotide/polymerase complex differ to those of the template-primer duplex/non-complementary nucleotide/polymerase complex.

According to the present invention, a method for sequencing a polynucleotide and/or identifying a single nucleotide polymorphism (SNP) comprises the steps of:

(i) localising a target polynucleotide duplex in space; and

(ii) reacting said duplex with at least one nucleotide in the presence of a polymerase enzyme under conditions sufficient for the polymerase reaction;

(iii) determining the whether or not said nucleotide is complementary to the target duplex polynucleotide via kinetic (temporal) analysis of the duplex/nucleotide/polymerase complex; and

(iv) adding a different nucleotide to the reaction and repeating steps i to iii in order to obtain sequence information.

Kinetic analysis can be out by measuring the temporal association of the target duplex/nucleotide/polymerase complex.

In a preferred embodiment of the invention, the polymerase is labelled such that its dwell time at the specific location of the target polynucleotide duplex can be measured and/or compared. In this preferred embodiment, the nucleotides used within the procedure are non-labelled. The use of non-labelled nucleotides has numerous advantages within the context of the present invention. A large number of array based sequencing procedures utilize labelled nucleotides in order to obtain the identity of the added base in sequencing by synthesis procedures. These assays rely on the stepwise identification of suitably labelled bases, referred to in US-A-5634413 as "single base" sequencing methods. The bases are incorporated onto the primer sequence using the polymerase reaction.

Such single base or sequencing by synthesis procedures usually utilise fluorescently labelled nucleotides in which the nascent chain (on the primer) is extended in a stepwise manner by the polymerase chain reaction. In these

situations each of the different nucleotides (A, T, G and C) incorporates a unique fluorophore at the 3' position which acts as a blocking group to prevent uncontrolled polymerisation. As used herein, the term "blocking group" refers to a moiety attached to a nucleotide which, while not interfering substantially with template-dependent enzymatic incorporation of the nucleotide into a polynucleotide chain, abrogates the ability of the incorporated nucleotide to serve as a substrate for further nucleotide addition. Such schemes employ a "removable blocking group". A "removable blocking group" is a blocking group that can be removed by a specific treatment that results in cleavage of the covalent bond between a nucleotide and the blocking group. The present invention has the advantage of negating the need for such "blocked nucleotides" or the need for "removable blocking groups". Such removable blocking groups rely on a number of possible removing treatment strategies. Specific treatments can be, for example, a photochemical, chemical or enzymatic treatment that results in the cleavage of the covalent bond between the nucleotide and fluorescent label. Such treatments, however, have been shown to be difficult to control and apply. Differences in local environments, with the context of an array, for example, could results in the removal of the entire nucleotide even several nucleotides inset of just the intended dye. Such occurrences obviously have serious consequences for the success of the sequencing method as uncontrolled removal of nucleotides results in sequencing data becoming out of phase and sequence date becoming corrupted or unusable.

The present invention, therefore, circumvents these problems by labelling the polynucleotide processive enzyme (i.e. polymerase) instead of the nucleotide itself. By removing the need for a label or nucleotide removal stage, the present invention drastically increases the practicality of "single base" sequencing methods.

Preferred embodiments of the present invention include fluorescence imaging techniques as well as raman scattering and surface electromagnetic wave (SEW) techniques such as surface plasmon resonance (SPR).

In a preferred embodiment, the enzyme is a polymerase enzyme which interacts with the target in the process of extending a complementary strand.

Description of the Invention

According to the present invention, a method for sequencing a polynucleotide and/or identifying a single nucleotide polymorphism (SNP) involves the temporal/kinetic analysis of the interaction between a polymerase enzyme, a target polynucleotide duplex and a nucleotide (complementary or non-complementary).

Measurement of the interaction of the polynucleotide processive enzyme, the template duplex and incoming nucleotide is preferably done with a label attached to the enzyme.

The term "polynucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridising nucleic acid-like molecules e.g. peptide nucleic acid (PNA).

The term "polynucleotide processive enzyme" as used herein is to be interpreted broadly and relates to any enzyme that interacts with a polynucleotide and moves continuously along the polynucleotide. The enzyme is preferably a polymerase enzyme, and may be of any known type. For example, the polymerase may be any DNA-dependent DNA polymerase. If the target polynucleotide is a RNA molecule, then the polymerase may be a RNA-dependent DNA polymerase, i.e. reverse transcriptase, or a RNA-dependent RNA polymerase, i.e. RNA replicase.

Alternative enzymes that interact with a polynucleotide include helicase, primase, holoenzyme, topoisomerase or gyrase enzymes.

The term "label" as used herein maybe interpreted broadly. Suitable labels will be apparent to the skilled person. In a preferred embodiment, the label is a fluorophore. Alternative labels may be used. A number of strategies for labelling molecules have been reported, such as microspheres (Anal. Chem. (2000) 72, 15:3678-3681), gold nanoparticles (J.Am.Chem. Soc. (2000) 122, 15:3795-3796), silver colloid particles (PNAS, (2000) 97, 3:996-1001) and quantum dots. Any labelling technique that allows unambiguous resolution of the kinetics of the polynucleotide processive enzyme's interaction with the template duplex/nucleotide complex (i.e. temporal resolution of enzyme binding/unbinding from polymer/nucleotide complex) can be utilized within the

context of the present invention.

In a preferred embodiment of the present invention, the template duplex of interest is localised in space via the immobilisation on a solid support. In is envisioned within the scope of the current invention that the immobilisation of the target polynucleotide may take a number of forms. It is envisioned that primers may be immobilised on the solid support and the target polynucleotide hybridised to them. It is also envisioned that hybridisation may take place in solution and the primer and/or target polynucleotide strand is then subsequently attached to a solid support. The envisioned "arrays" of target polynucleotide duplexes may be of any known density, including multi-molecule high-density arrays as well as "single-molecule" arrays in which individual polynucleotide locations may be resolved.

In a preferred embodiment of the present invention, fluorescent dyes are attached to the polynucleotide processive enzyme. Once an array of immobilised target polynucleotide duplexes has been created, the labelled polynucleotide processive enzyme is flowed over the array (within a flow cell) in conditions sufficient for enzyme activity with one or several of the four nucleotides present in solution. Each "array location" within the array is then imaged at a time resolution that allows resolution of the kinetic binding of the polymerase to the polynucleotide-duplex/nucleotide complex (i.e. from 1 to several thousand hertz at least).

In a preferred embodiment of the present invention, only one nucleotide at a time is flowed over the immobilised template duplex with the labelled polynucleotide processive enzyme. The "dwell" time of the labelled enzyme at the array location is then used to determine whether or not the nucleotide added in solution with the enzyme is complementary or non-complementary. Non-reacted nucleotides are then removed from the array (i.e. via a wash step/cycle within the flow cell). Generally speaking, non-complementary nucleotides will result in a enzyme dwell time shorter than that of complementary nucleotides (a preferred embodiment of the present invention is that the reaction is carried out within a flow stream). Further, the addition of two or more of the same nucleotide with result in an increase of the enzymatic dwell time by a similar factor. This

information can thus be calculated by a computer program programmed with appropriate kinetic information in order to build up a nucleotide sequence at the location of each target polynucleotide duplex.

In a further preferred embodiment of the present invention, the polynucleotide processive enzyme is labelled with a metal sphere or other raman and/or plasmon supporting structure. In the context of the present invention, therefore, raman scattering/shift is monitored at sufficient temporal resolution (to obtain kinetic data) at each polynucleotide-duplex reaction/array site. The dwell time and/or raman spectral shift of the polynucleotide processive enzyme (with the raman particle attached) is then measured and used to determine the existence of complementary on non-complementary nucleotide within solution and the base sequence built up as before (base by base sequencing).

In a preferred embodiment the raman shifts of from the labelled or non-labelled polynucleotide process enzyme only is measured in order to determine whether or not the base added in solution with the enzyme is complementary or non-complementary.

In another preferred embodiment, the polynucleotide processive enzyme is labelled with a fluorescence resonance energy transfer pair (FRET) pair on the same molecule. These fluorophores are chosen so the emission wavelength of one of the dyes corresponds to the excitation wavelength of the other (classical FRET pairs). The immobilisation locations on the molecules are chosen such that they move relative to one another when the enzyme binds to a polynucleotide complex, preferably upon binding to a polynucleotide/nucleotide complex. This relative movement between FRET pairs will result in a spectral shift of the emission from the FRET pair (depending upon the way in which the FRET pair has been designed). This will mean that either more or less energy is coupled into the acceptor pair from the donor leading to attenuation of the donor fluorescence. In a further preferred embodiment, acceptor and/or donor fluorescence in monitored with high temporal resolution (in order to obtain kinetic information) and with appropriate spatial resolution (depending upon the array density used). In a preferred embodiment of the invention, the imaging device used in a charge-coupled device (CCD) with high read-out time and appropriate

optics for high spatial resolution.

In a further preferred embodiment, the template duplex is immobilised on a solid support able to sustain a surface electromagnetic wave (SEW). This surface wave may be used to excite the fluorophores and/or raman scattering in the aforementioned embodiments. In a preferred embodiment of the present invention the SEW is an evanescent wave.

In a further preferred embodiment of the present invention, the SEW is a surface plasmon wave. In such an embodiment, the polynucleotide processive need not be "labelled". In a preferred embodiment, the enzyme is a polymerase, which is in its native form and the aforementioned temporal/kinetic monitoring is carried out via surface plasmon resonance (i.e. change in relative dielectric constant). Binding of the polymerase to the target duplex will change the relative dielectric constant of the space and immediate vicinity occupied by the immobilised polynucleotide.

In yet another preferred embodiment of the present invention, kinetic monitoring of polymerase binding using surface plasmon resonance may be enhanced by adding a "label" to the polymerase with a large dielectric constant. Suitable labels include metal particles and plasmon supporting nanoparticles. Such particles may be attached to the polymerase via a number of procedure well known in the art.

Claim

1. A method for sequencing a polynucleotide and/or identifying a single nucleotide polymorphism (SNP) comprises the steps of:

(i) localising a target polynucleotide duplex in space; and

(ii) reacting said duplex with at least one nucleotide in the presence of a polymerase enzyme under conditions sufficient for the polymerase reaction;

(iii) determining the whether or not said nucleotide is complementary to the target duplex polynucleotide via kinetic (temporal) analysis of the duplex/nucleotide/polymerase complex; and

(iv) adding a different nucleotide to the reaction and repeating steps i to iii in order to obtain sequence information.

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